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(21) International Application Number: PCT/AU98/00730 (22) International Filing Date: 8 September 1998 (08.09.98) (30) Priority Data: PO 9002 8 September 1997 (08.09.97) AU (71) Applicant (for all designated States except US): MEDVET SCIENCE PTY. LTD. [AU/AU]; Hanson Centre, Level 3, IMVS, Frome Road, Adelaide, S.A. 5000 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): VADAS, Mathew [AU/AU]; 8 Branch Road, Stirling, S.A. 5152 (AU). GAMBLE, Jennifer [AU/AU]; 8 Branch Road, Stirling, S.A. 5152 (AU). XIA, Pu [CN/AU]; 1/225 Beulah Road, Beulah Park, S.A. 5067 (AU). BARTER, Philip [AU/AU]; 49 Ormond Grove, Toorak Gardens, S.A. 5065 (AU). RYE, Kerry-Anne [AU/AU]; 49 Ormond Grove, Toorak Gardens, S.A. 5065 (AU). WATTENBERG, Brian [US/AU]; 137 Sheoak Road, Belair, S.A. 5052 (AU). PITSON, Stuart [AU/AU]; 4/19 Desaumarez Street, Kensington Park, S.A. 5068 (AU). (74) Agents: SLATTERY, John, M. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: A METHOD OF MODULATING CELLULAR ACTIVITY (57) Abstract <p>The present invention relates generally to a method of modulating cellular activity and agents useful for same. More particularly, the present invention contemplates a method of modulating endothelial cell activity and even more particularly endothelial cell adhesion molecule expression. Most particularly, the present invention provides a method of treating coronary heart disease by preventing or reducing endothelial cell adhesion molecule expression.</p>		

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A METHOD OF MODULATING CELLULAR ACTIVITY

FIELD OF THE INVENTION

5 The present invention relates generally to a method of modulating cellular activity and agents useful for same. More particularly, the present invention contemplates a method of modulating endothelial cell activity and even more particularly endothelial cell adhesion molecule expression. Most particularly, the present invention provides a method of treating coronary heart disease by preventing or reducing endothelial cell adhesion molecule
10 expression.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected
15 at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined immediately before the bibliography.

Atherosclerotic coronary heart disease is one of the major causes of death in the western world
20 (World Health Statistics Annual). An earlier event in atherogenesis is the adhesion of monocytes to the endothelium via adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, all of which are rapidly synthesised in response to cytokines. VCAM-1 is primarily involved in the adhesion of mononuclear leukocytes to the endothelium. It is rapidly induced by the inflammatory cytokines IL-1 and TNF- α , and its induction is sustained for 48 to 72 hours.
25 ICAM-1 is expressed on many cells types and is involved in both monocyte and lymphocyte adhesion to activated endothelium. E-selectin is an endothelial specific adhesion molecule important in capturing leukocytes from the axial stream to roll along the endothelium (Abbassi *et al.*, 1993).

30 There is considerable evidence for the involvement of adhesion molecules in the development of early atherosclerotic lesions and in mature atherosclerotic plaques (Van der Wal *et al.*, 1992).

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Variable and low levels of E-selectin and VCAM-1 have been detected in the arterial endothelium over plaques (Van der Wal *et al.*, 1992; Wood *et al.*, 1993). VCAM-1 has also been observed in areas of neovascularization and in inflammatory infiltrates at the base of plaques, suggesting that intimal neovascularization may be an important site of inflammatory cell
5 recruitment into advanced coronary lesions (O'Brien *et al.*, 1993). ICAM-1 has been shown to be expressed on the endothelium overlaying atheromatous plaques (Johnson-Tidey *et al.*, 1994).

The signals that lead to upregulation of cellular activities such as expression of adhesion molecules have not been defined. Elucidating these cellular signalling mechanisms is necessary
10 for the development of therapeutic strategies to disease conditions in which said cellular activities are harmful such as coronary heart disease and inflammatory conditions.

In work leading up to the present invention, the inventors have identified a sphingosine kinase signalling pathway via which cellular activities such as adhesion molecule expression are
15 achieved. By regulating the expression and activity of individual components of this pathway, these cellular activities can be modulated. The inventors have also developed a rapid, high volume assay for detecting agents exhibiting sphingosine kinase activity and agents which can act as agonists and antagonists of sphingosine kinase activity.

20 SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps
25 but not the exclusion of any other integer or step or group of integers or steps.

One aspect of the present invention relates to a method of modulating cellular activity in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components
30 of a sphingosine kinase signalling pathway.

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Another aspect of the present invention provides a method of modulating adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway.

5

Yet another aspect of the present invention provides a method of modulating adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or both of sphingosine kinase and/or Sph-1-P.

10

Still another aspect of the present invention relates to a method of modulating endothelial cell adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway.

15

Still yet another aspect of the present invention provides a method of downregulating endothelial cell adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to downregulate the activity of one or both of sphingosine kinase and/or Sph-1-P.

20

A further aspect of the present invention relates to a method of modulating adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof.

25

Another further aspect of the present invention relates to a method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway

30 wherein said modulation results in modulation of adhesion molecule expression.

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Still another further aspect of the present invention provides a method of treating a mammal exhibiting coronary heart disease said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to downregulate the activity of one or more components of a sphingosine kinase signalling pathway wherein said
5 downregulation results in downregulation of endothelial cell adhesion molecule expression.

Still yet another further aspect of the present invention provides a method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms said method comprising administering an effective amount of one or more components of a sphingosine kinase signalling
10 pathway or functional equivalents thereof to said mammal.

In still yet another further aspect the present invention relates to the use of an agent capable of modulating the activity of one or more components of a sphingosine kinase signalling pathway in the manufacture of a medicament for the modulation of adhesion molecule
15 expression in a mammal.

Another aspect of the present invention relates to the use of one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof in the manufacture of a medicament for the modulation of adhesion molecule expression in a mammal.
20

Yet another aspect of the present invention relates to agents for use in modulating one or more components of a sphingosine kinase signalling pathway wherein modulating said components modulates adhesion molecule expression.

25 Yet another aspect of the present invention relates to one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof for use in modulating adhesion molecule expression in a mammal.

Still another aspect of the present invention relates to a pharmaceutical composition
30 comprising an agent capable of modulating one or more components of a sphingosine kinase signalling pathway wherein said modulation results in modulation of adhesion molecule

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expression, together with one or more pharmaceutically acceptable carriers and/or diluents.

Still yet another further aspect of the present invention relates to a pharmaceutical composition comprising one or more components of a sphingosine kinase signalling pathway
5 or functional equivalents thereof together with one or more pharmaceutically acceptable carriers and/or diluents.

A further aspect of the present invention provides a method of detecting an analyte structurally or functionally reactive with a lipid, said method comprising the steps of
10 contacting either:

- (i) said analyte which analyte is radio labelled; or
- (ii) said analyte and a reporter molecule which reporter molecule is radio labelled,
15 said analyte being contacted with said lipid simultaneously with or separately to said reporter molecule;

with said lipid in the presence of a scintillant for a time and under conditions sufficient for a lipid-radio label complex to form and to excite said scintillant, and detecting said excited
20 scintillant.

Another further aspect of the present invention relates to a method of detecting an analyte structurally or functionally reactive with sphingosine, said method comprising the steps of contacting either:

- (i) said analyte which analyte is radio labelled; or
- (ii) said analyte and a reporter molecule which reporter molecule is radio labelled,
25 said analyte being contacted with said sphingosine simultaneously with or separately to said reporter molecule;
30

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with said lipid in the presence of a scintillant for a time and under conditions sufficient for a sphingosine-radio label complex to form and to excite said scintillant, and detecting said excited scintillant.

- 5 Still another further aspect of the present invention provides a method for detecting an analyte exhibiting sphingosine kinase activity, said method comprising the steps of contacting said analyte and ^{33}P -ATP with sphingosine in the presence of a scintillant, said analyte being contacted with said sphingosine simultaneously with or separately to said ^{33}P -ATP, for a time and under conditions sufficient for ^{33}P -sphingosine complex to form and to excite said
10 scintillant, and detecting said excited scintillant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphical representation of the effect of high density lipoprotein (HDL) on tumour
15 necrosis factor (TNF) mediated induction of adhesion proteins VCAM-1 and E-selectin. Mean fluorescence intensity is a flow cytometric measurement signifying the intensity of expression of adhesion molecules on the cell surface.

Figure 2 is a graphical representation of the effect of sphingosine kinase inhibitor on adhesion
20 protein expression. Intensity of cell surface expression was measured as a percent of the mean fluorescence intensity stimulated by 100U of TNF. Figure 2A filled symbols show the effect of the cell-permeable ceramide (C2-ceramide) on adhesion proteins. The open symbols show the effect of ceramide in the presence of 100U/ml TNF. In Figure 2B the effect of sphingosine-1-phosphate (Sph-1-P) is shown. Figure 2C shows the effect dimethylsphingosine (DMS) on
25 adhesion protein expression (MFI) stimulated by TNF or Sph-1-P. In Figure 2D the effect of these agents on E-selectin mRNA is shown.

Figure 3 is a graphical representation of the time course of changes after TNF treatment of HUVECs on key components of sphingomyelin pathway are shown. Open symbols in Figure 3A
30 show hydrolysis of sphingomyelin, in Figure 3B generation of ceramide, in Figure 3C sphingosine kinase activity and in Figure 3D formation of sphingosine-1-phosphate, all as percent of amount

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at time 0. Filled circles represent the effect of pre-treatment of HUVECs with HDL.

Figure 4 is a photographic representation of the effect of Sph-1-P, TNF and HDL on generation of ERK (Figure 4A) and NF- κ B (Figure 4B). The bar graph provides a summary of three independent experiments on ERK activation (\pm 1 SEM). * show p value of difference from TNF by <0.01 and \dagger by <0.05 , \ddagger shows p <0.01 versus nil counted. Figure 4B top shows gel retardations with NF- κ B. Figure 4B bottom shows supershifts with antibodies specific for p50 and p65 components of NF- κ B and demonstrates the similar composition of NF- κ B stimulated by TNF and Sph-1-P.

10

Figure 5 is a schematic representation of the sphingosine kinase pathway by which HDL inhibits TNF α -induced adhesion protein expression.

Figure 6 is a schematic representation of the multi-well assay for sphingosine-kinase activity. The basis of the assay is the phosphorylation of sphingosine bound by absorption to the walls of a multi-well plate in which the walls have been impregnated with scintillant (Flashplates, New England Nuclear). γ - ^{33}P labelled ATP is incubated in the presence of the source of enzyme. If active enzyme is present the ^{33}P is transferred to the sphingosine. The wells are then washed to remove unbound ^{33}P . The ^{33}P bound to the sphingosine excites the scintillant and a signal is produced that is measured in a scintillation counter.

Figure 7 is a graphical representation showing that sphingosine kinase activity is linear for up to 400 minutes when measured in the multi-well assay. 50 μl of an endothelial cell extract (prepared as described above in example 8, containing sphingosine kinase activity) plus 50 μl of an ATP solution containing 400 μM ATP/20 $\mu\text{Ci/ml}$ ^{33}P -ATP was incubated with flashplates coated with phospholipid mixes either with (filled squares) or without (open squares) sphingosine and incubated at 37°C for the indicated lengths of time before washing.

Figure 8 is a graphical representation of a survey of tissues for sphingosine-kinase activity. High speed "supernatant" (open bar) and "pellet" (filled bar) fractions from 50 μl of extract were then assayed for sphingosine kinase activity.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Accordingly, one aspect of the present invention relates to a method of modulating cellular
5 activity in a mammal said method comprising administering to said mammal an effective amount
of an agent for a time and under conditions sufficient to modulate the activity of one or more
components of a sphingosine kinase signalling pathway.

The term "mammal" includes humans, primates, livestock animals (e.g. horses, cattle, sheep, pigs,
10 donkeys), laboratory test animals (e.g. mice, rats, rabbits, guinea pigs), companion animals (e.g.
dogs and cats) and captive wild animals (e.g. kangaroos, deer, foxes). Preferably, the mammal
is a human or a laboratory test animal. Even more preferably the mammal is a human.

Reference to "modulating cellular activity" is a reference to up-regulating, down-regulating or
15 otherwise altering any one or more of the activities which a cell is capable of performing such
as, but not limited to, one or more of chemokine production, cytokine production, nitric oxide
synthetase production, adhesion molecule expression and production of other inflammatory
modulators. Preferably said cellular activity is adhesion molecule expression. Reference
hereinafter to adhesion molecule expression should be read as including reference to other
20 cellular activities.

Accordingly, there is provided a method of modulating adhesion molecule expression in a
mammal said method comprising administering to said mammal an effective amount of an agent
for a time and under conditions sufficient to modulate the activity of one or more components
25 of a sphingosine kinase signalling pathway.

The term "expression" refers to the transcription and translation of a nucleic acid molecule
resulting in the synthesis of a polypeptide.

30 Reference to "adhesion molecule" should be understood as a reference to a molecule which
mediates the binding of a cell to another cell or to a protein such as an extracellular matrix

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protein. Examples of adhesion molecules include, but are not limited to, integrins, selectins (e.g. E-selectin, P-selectin), members of the immunoglobulin-gene superfamily (e.g. VCAM-1, ICAM-1) and CD44.

5 Reference to modulating the "activity" of sphingosine kinase signalling pathway components is a reference to modulating the functions which said components are capable of performing such as but not limited to increasing or decreasing the role or extent to which a given component performs its function or modifying the nature of the function which a given component performs. Modulating said "activity" should also be understood to encompass increasing or decreasing the
10 concentration levels of a given component. Modulation of said "activity" may be achieved by:

- (i) modulation of the catalytic activity of sphingosine kinase by competition with substrate (for example, sphingosine or ATP);
- 15 (ii) interference with the catalytic activity of sphingosine kinase by an allosteric mechanisms (binding to sites on the molecule other than the substrate-binding sites); or
- (iii) interfering with enzyme activation, such as by altering:
 - 20 - post-translational covalent modification such as phosphorylation, lipid modification
 - non-covalent coupling to a required co-activator such as a protein, lipid or ion
 - subcellular localisation of the enzyme.

25 A "sphingosine kinase signalling pathway" is defined as a signalling pathway which utilises one or both of sphingosine kinase and/or sphingosine-1-phosphate. Without limiting the present invention to any one theory or mode of action, it is thought a sphingosine kinase signalling pathway cascade which results in adhesion molecule expression may take the form of:

- 30 (i) the generation of ceramide from sphingomyelin via *S.Mase* activity, said ceramide being converted to sphingosine;

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(ii) sphingosine-1-phosphate (referred to hereinafter as "Sph-1-P") generation by stimulation of sphingosine kinase; and

5 (iii) the activation of MEK/ERK and nuclear translocation of NF- κ B downstream from Sph-1-P generation, said downstream events leading to adhesion molecule expression.

Accordingly, the term "components" should be understood to refer to any molecule which is or may be involved in a sphingosine kinase signalling pathway cascade and includes, but is not
10 limited to, cellular proteins, metabolites (e.g. sphingomyelin, ceramide, sphingosine and Sph-1-P), kinases (e.g. *S.Mase*, sphingosine kinase, protein kinase C and ERK) and transcription factors (e.g. NF- κ B). Preferably, said components are sphingosine kinase and/or Sph-1-P.

According to this preferred embodiment there is provided a method of modulating adhesion
15 molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or both of sphingosine kinase and/or Sph-1-P.

A key event in many disease conditions is the upregulation of cellular activities such as those
20 which lead to inflammation. For example, upregulation of the production of inflammatory mediators such as cytokines, chemokines, eNOS and upregulation of adhesion molecule expression. Said upregulation may be induced by a number of stimuli including, for example, inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and interleukin-1 (IL-1), endotoxin, oxidised or modified lipids, radiation or tissue injury. In a preferred aspect said
25 cellular activity is endothelial cell activity.

Accordingly, the present invention relates to a method of modulating endothelial cell adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity
30 of one or more components of a sphingosine kinase signalling pathway.

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Most preferably said components are sphingosine kinase and/or Sph-1-P.

While most of the components involved in a sphingosine kinase signalling pathway are stimulatory for a given cellular activity, inhibitory signalling components are also evident. For example, Sph-1-P is generated from sphingosine by sphingosine kinase. Stimulation of endothelial cells causes a rapid and transient increase in cytosolic sphingosine kinase activity which leads to upregulation of adhesion molecule expression. The production of Sph-1-P is induced in parallel with sphingosine kinase activity. However, inhibitory components of a sphingosine kinase signalling pathway result in inhibition of adhesion molecule expression. Accordingly, the term "modulate" in relation to the activity of any one or more components of the sphingosine kinase signalling pathway refers to upregulating or downregulating or otherwise altering said activity. The preferred method is to downregulate sphingosine kinase pathway activity either by inhibiting or reducing the activity of one or more stimulatory components of a sphingosine kinase signalling pathway or by upregulating the activity of one or more inhibitory components of said pathway. However, modulation of the activity of said components wherein adhesion molecule expression is upregulated may be desired under certain circumstances. Most preferably, the biological activity of said stimulatory components is down-regulated.

According to this most preferred embodiment, there is provided a method of downregulating endothelial cell adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to downregulate the activity of one or both of sphingosine kinase and/or Sph-1-P.

Modulation of said activity by the administration of an agent to a mammal can be achieved by one of several techniques, including but in no way limited to introducing into said mammal a proteinaceous or non-proteinaceous molecule which:

- (i) modulates synthesis of said components;
- (ii) functions as an antagonist to said component;

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(iii) functions as an agonist to said component.

Said proteinaceous molecule may be derived from natural, recombinant or synthetic sources including fusion proteins or following, for example, natural product screening. Said non-
5 proteinaceous molecule may be derived from natural sources, such as for example natural product screening or may be chemically synthesised. The present invention contemplates chemical analogs of said components capable of acting as agonists or antagonists of said components. Chemical agonists may not necessarily be derived from said components but may share certain conformational similarities. Alternatively, chemical agonists may be specifically
10 designed to mimic certain physiochemical properties of said components. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing said components from carrying out their normal biological functions. Antagonists include monoclonal antibodies specific for said components, or parts of said components, and antisense nucleic acids which prevent transcription or translation of genes or mRNA in mammalian cells.

15

The method of the present invention is exemplified herein utilising high density lipoprotein (referred to herein as "HDL"). Treatment of endothelial cells with HDL inhibits both the amplitude and duration of TNF- α induced sphingosine kinase activation. HDL treatment substantially blunts the amplitude and duration of Sph-1-P formation by inhibiting sphingosine
20 kinase activity. The inhibition of Sph-1-P formation results in blunting of downstream pathway events including blunting of MEK/ERK activation and NF- κ B nuclear translocation thereby reducing adhesion protein expression. Similarly, N,N-dimethyl sphingosine decreases TNF- α induced adhesion protein expression and mRNA levels by competitively inhibiting sphingosine kinase activity. Interruption of the sphingosine kinase signalling pathway at this point prevents
25 the downstream formation of intracellular Sph-1-P thereby preventing the further downstream events of MEK/ERK and NF- κ B stimulation which lead to adhesion protein expression.

In a most preferred embodiment the present invention relates to a method of downregulating endothelial cell adhesion molecule expression in a mammal said method comprising administering
30 to said mammal an effective amount of HDL for a time and under conditions sufficient to downregulate sphingosine kinase activity.

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An "effective amount" means an amount necessary to at least partly obtain the desired response or to delay the onset or inhibit progression or halt altogether the onset or progression of adhesion molecule expression. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated, the assessment
5 of the medical situation and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Modulation of cellular adhesion molecule expression can also be achieved by the administration of one or more sphingosine kinase signalling pathway components or functional equivalents
10 thereof.

Accordingly, another aspect of the present invention relates to a method of modulating adhesion molecule expression in a mammal said method comprising administering to said mammal an effective amount of one or more components of a sphingosine kinase signalling pathway or
15 functional equivalents thereof.

The term "functional equivalents" used herein includes but is not limited to derivatives having the functional activity of said components.

20 Derivatives include fragments, parts, portions, chemical equivalents, mutants, homologs, mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence
25 variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted
30 in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

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The derivatives of said components include fragments having particular epitopes or parts of the entire component fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, said components or derivative thereof may be fused to a molecule to facilitate its entry into a cell. Analogs of said components contemplated
5 herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogs. Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions
10 including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in cosuppression and fusion of nucleic acid molecules.

Examples of side chain modifications contemplated by the present invention include
15 modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with
20 pyridoxal-5-phosphate followed by reduction with NaBH_4 .

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

25 The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed
30 disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other

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substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

- 5 Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.
- 10 Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carboethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during protein synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-
15 phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid contemplated herein is shown in Table 1.

20

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TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
10 aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine		L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
15 D-alanine	Dal	Chexa L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Das	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
20 D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
25 D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
30 D-threonine	Dthr	L-N-methylethylglycine	Nmetg
		L-N-methyl-t-butylglycine	Nmtbug
		L-norleucine	Nle

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	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgab
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
5	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methylleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
30	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser

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	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methyllleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
5	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
10	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
15	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylassparagine	Masn
	L- α -methylasspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
20	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomophenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L- α -methyllleucine	Mleu	L- α -methyllysine	Mlys
	L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
25	L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
	L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
	L- α -methylserine	Mser	L- α -methylthreonine	Mthr
	L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
	L- α -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhph

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N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
carbamylmethyl)glycine		carbamylmethyl)glycine	
1-carboxy-1-(2,2-diphenyl-	Nmbc		
ethylamino)cyclopropane			

5

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with $n=1$ to $n=6$, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional

10 reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_ϵ -methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues

15 by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The present invention is useful in relation to human disease conditions. For example the present invention is particularly useful, but in no way limited to, use as a prophylactic or as a therapy

20 in relation to disease conditions which involve inflammatory mechanisms, such as coronary heart disease.

Accordingly, another aspect of the present invention relates to a method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms in a mammal said

25 method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway wherein said modulation results in modulation of adhesion molecule expression.

30 Preferably modulation of said adhesion molecule expression is modulation of endothelial cell adhesion molecule expression.

- 20 -

Even more preferably, said components are sphingosine kinase and/or Sph-1-P. Most preferably said components are downregulated said downregulation resulting in downregulation of adhesion molecule expression.

5 In a most preferred embodiment there is provided the method of treating a mammal exhibiting coronary heart disease said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to downregulate the activity of one or more components of a sphingosine kinase signalling pathway wherein said downregulation results in downregulation of endothelial cell adhesion molecule expression.

10

Most preferably said components are sphingosine kinase and/or Sph-1-P.

Another aspect of the present invention provides a method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms in a mammal said method comprising
15 administering to said mammal an effective amount of one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof wherein said modulation results in modulation of adhesion molecule expression.

Administration of the agent or component or functional equivalent thereof, in the form of
20 a pharmaceutical composition, may be performed by any convenient means. The agent or component or functional equivalent thereof of the pharmaceutical composition is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the agent chosen. A broad range of doses may be applicable. Considering a patient, for
25 example, from about 0.1 mg to about 1 mg of the agent or component or functional equivalent thereof may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or at other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.
30 The agent or component or functional equivalent thereof may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal,

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intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of the agent or component or functional equivalent thereof, said agent or component or functional equivalent thereof may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

In a preferred embodiment of the present invention, the agent used in the method is linked to an antibody specific for coronary endothelial cells to enable specific delivery of said agent to these cells.

In yet another aspect the present invention relates to the use of an agent capable of modulating the activity of one or more components of a sphingosine kinase signalling pathway in the manufacture of a medicament for the modulation of adhesion molecule expression in a mammal.

Preferably modulation of said adhesion molecule expression is modulation of endothelial cell adhesion molecule expression.

Even more preferably, said components are sphingosine kinase and/or Sph-1-P. Most preferably said components are downregulated said downregulation resulting in downregulation of adhesion molecule expression.

Another aspect of the present invention relates to the use of one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof in the manufacture of a medicament for the modulation of adhesion molecule expression in a mammal.

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Yet another aspect of the present invention relates to agents for use in modulating one or more components of a sphingosine kinase signalling pathway wherein modulating said components modulates adhesion molecule expression.

- 5 Preferably modulation of said adhesion molecule expression is modulation of endothelial cell adhesion molecule expression.

Even more preferably, said components are sphingosine kinase and/or Sph-1-P. Most preferably said components are downregulated said downregulation resulting in downregulation
10 of adhesion molecule expression.

A further aspect of the present invention relates to one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof for use in modulating adhesion molecule expression in a mammal.

15

Yet another further aspect of the present invention relates to a pharmaceutical composition comprising an agent capable of modulating one or more components of a sphingosine kinase signalling pathway wherein said modulation results in modulation of adhesion molecule expression, together with one or more pharmaceutically acceptable carriers and/or diluents.

20

In another further aspect, the present invention relates to a pharmaceutical composition comprising one or more components of a sphingosine kinase signalling pathway or functional equivalents thereof together with one or more pharmaceutically acceptable carriers and/or diluents.

25

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and
30 storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for

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example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of
5 surfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying
10 absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared
15 by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously
20 sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be
25 incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied
30 and may conveniently be between about 5 to about 80% of the weight of the unit. The

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amount of active compound in such therapeutically useful compositions in such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μ g and 2000 mg of active compound.

5

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or
10 saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain
15 the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

20

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating the expression of a component of the sphingosine kinase signalling pathway. The vector may, for example, be a viral vector.

25

To facilitate the identification of agents suitable for use in the method of present invention, the inventors have developed a rapid, facile, high volume assay for screening for agents which are either structurally or functionally reactive with a lipid. In the context of identifying agents for use in the method of the present invention, this assay provides a means
30 of easily and rapidly identifying agents which either mimic the activity of a component of

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sphingosine kinase signalling pathway or act as an agonist or antagonist to a component of sphingosine kinase signalling pathway.

Accordingly, another aspect of the present invention provides a method of detecting an
5 analyte structurally or functionally reactive with a lipid, said method comprising the steps of contacting either:

- (i) said analyte, which analyte is radio labelled; or
- 10 (ii) said analyte and a reporter molecule which reporter molecule is radio labelled, said analyte being contacted simultaneously with or separately to said reporter molecule;

with said lipid in the presence of a scintillant for a time and under conditions sufficient for
15 a lipid-radio label complex to form and to excite said scintillant, and detecting said excited scintillant.

Preferably said analyte or analyte and reporter molecule are contacted with lipid and scintillant coupled to a solid support.

20

Reference to "lipid" should be understood as a reference to any molecule comprising a lipid component. Preferably said lipid is a lipid component of the sphingosine kinase signalling pathway and even more preferably sphingosine. This aspect of the present invention is exemplified herein with respect to sphingosine, however this should not be understood as
25 imposing any limitation on the application of this method.

Accordingly, the present invention more particularly provides a method of detecting an analyte structurally or functionally reactive with sphingosine, said method comprising the steps of contacting either:

30

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- (i) said analyte which analyte is radio labelled; or
- (ii) said analyte and a reporter molecule which reporter molecule is radio labelled, said analyte being contacted simultaneously with or separately to said reporter molecule;

5

with said sphingosine in the presence of a scintillant for a time and under conditions sufficient for a sphingosine-radio label complex to form and to excite said scintillant, and detecting said excited scintillant.

10

Preferably, said analyte or analyte and reporter molecule are contacted with sphingosine and scintillant which sphingosine may be coupled to a solid support having a scintillant already coupled thereto using a method as described in Example 9.

- 15 Reference to an analyte which is "structurally" reactive with a lipid should be understood as a reference to a molecule which either binds, links or otherwise associates with said lipid. Said association may be due to the formation of a peptide bond, ionic bond, hydrogen bond or other interactive bonding mechanisms. Reference to an analyte which is "functionally" reactive with a lipid should be understood as a molecule which, when it interacts with said
- 20 lipid, directly or indirectly causes a functional process to take place. A functionally reactive analyte may or may not also structurally interact with said lipid. An example of a functionally reactive analyte is an enzyme (for example, a sphingosine kinase equivalent) the substrate of which is a lipid (for example, sphingosine). In accordance with this example, an enzyme exhibiting sphingosine kinase activity is functionally reactive in that it will
- 25 phosphorylate sphingosine in the presence of ATP. The occurrence of the structural or functional activity is detected by the method of this aspect of the present invention.

The detection of functional or structural reactivity is based on the formation, by the lipid, of a radio labelled complex. Due to the proximity of the complexed radio label with the

30 scintillant, the scintillant becomes excited.

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The analyte which is being assayed may be directly radio labelled. This is useful where, for example, said analyte directly associates with said lipids. Alternatively, a reporter molecule may be radio labelled. Reference to "reporter molecule" should be understood as a reference to any molecule, other than the analyte, which detects reactivity between the analyte and
5 lipid. For example, the reporter molecule may be an antibody which recognises one or more epitopes present on the surface of the analyte of interest. This method of detection is an indirect method of detection, commonly referred to as a "sandwich assay". Alternatively, the reporter molecule may be a molecule required to both facilitate reactivity between the analyte and the lipid, for example the reporter molecule may be a substrate, in addition to
10 facilitating detection. In the method exemplified herein, the reporter molecule is ^{33}P -ATP. An analyte exhibiting sphingosine kinase activity will phosphorylate sphingosine in the presence of ^{33}P -ATP resulting in the coupling of ^{33}P to the sphingosine. In the absence of a phosphate reporter molecule, the analyte would be unable to phosphorylate the lipid. Further, since the analyte does not structurally interact with the lipid sufficiently to facilitate
15 detection, the method of detection of the functional reactivity is based on the phosphorylation of the lipid with radio labelled phosphate.

It should be understood, that even where the analyte is radio labelled and said analyte binds to the lipid, it may nevertheless be necessary that a molecule other than the analyte is present
20 in the reaction mixture to facilitate reactivity of the analyte with the lipid.

Solid supports suitable for use in the present invention include, but are not limited to, microtitre plates, 96 well plates, columns and micro-beads.

25 Preferably, the present invention provides a method for detecting an analyte exhibiting sphingosine kinase activity, said method comprising the steps of contacting said analyte and ^{33}P -ATP with sphingosine in the presence of a scintillant, said analyte being contacted with said sphingosine simultaneously with or separately to said ^{33}P -ATP, for a time and under conditions sufficient for ^{33}P -sphingosine complex to form and to excite said scintillant, and
30 detecting said excited scintillant.

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Preferably said analyte and ^{33}P -ATP are contacted with sphingosine and scintillant coupled to a solid support.

Detection of the excited scintillant may be by any suitable means including, but not limited to, a scintillation counter.

The analyte which is assayed in the method of this aspect of the present invention may be in any suitable form. A fluid form is particularly useful as are tissue extracts such as lysates or homogenates. Said analyte may be a proteinaceous or a non-proteinaceous molecule derived from natural, recombinant or synthetic sources.

In another aspect, the method of the present invention should be understood to extend to the detection of agents which act as agonists or antagonists of analytes which either structurally or functionally react with a lipid. For example, the present invention is particularly useful for detecting agonists or antagonists of sphingosine kinase activity. This may be achieved, in accordance with this embodiment of the present invention, by contacting the sphingosine with sphingosine kinase in the presence of the potential agonistic or antagonistic agent. Comparison of the degree of sphingosine-radio label complex formation relative to a control assay in which addition of said agent was omitted is indicative of agonistic or antagonistic agent activity. Specifically, an increase in sphingosine ^{33}P complex formation relative to the control is indicative of agonistic activity while a decrease in sphingosine ^{33}P complex activity is indicative of antagonistic activity. This aspect of the present invention should not in any way be taken as limited to the embodiment described above. Rather this aspect of the present invention can be applied to detect antagonists or agonists of any analyte which either structurally or functionally react with a lipid.

Agents suitable for testing in the method of the present invention include, but are not limited to compounds isolated from chemical libraries or broths produced from fermentation of an organism.

30

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With respect to the preferred embodiment detailed above, sources of sphingosine kinase suitable for use in the method of this aspect of the present invention include, but are not limited to: (i) partially purified sphingosine kinase derived from mammalian tissue, (ii) crude homogenates of mammalian tissue exhibiting enzyme activity, (iii) recombinant enzyme or
5 (iv) HUVEC crude lysate.

Further features of the present invention are more fully described in the following examples. It is to be understood, however, that this detailed description is included solely for the purposes of exemplifying the present invention. It should not be understood in any way as
10 a restriction on the broad description of the invention as set out above.

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EXAMPLE 1**HDL isolation**

HDL were isolated from normal healthy adult donors by sequential ultracentrifugation in the density range of 1.07-1.21 g/ml. Resulting preparation of HDL contained two main populations: one with particles of Stokes' diameter 10.45 nm (HDL₂) and one with particles of diameter 8.6 nm (HDL₃). Since the inhibition of adhesion molecules expression of HDL₃ was stronger than that induced by other native or reconstituted HDL particles from purified Apo-A-1 and egg phosphatidylcholin or sphingomyelin, HDL₃ was thus used in this study.

10 In the examples we have used the human HDL, subfraction (d=1.13~1.21 g/ml) in order to minimise possible confounding effects of variations in the relative proportions of HDL₂ and HDL₃ in the plasma of different subjects. As shown in Figure 1 HDL₃ inhibited the TNF α -induced expression of VCAM-1 and E-selectin by human umbilical vein endothelial cells (HUVECs) by >80%.

15

EXAMPLE 2**Sphingosine kinase signalling pathway methodology***Effect of HDL on TNF α -induced adhesion protein expression by HUVECs*

20

Confluent monolayers of HUVECs were preincubated with or without HDL that was isolated as the fraction of density 1.13~1.21 g/ml from human plasma at a concentration of 1 mg/ml of apoA-1. After 16-h preincubation, the cells were treated with TNF α (100 U/ml). Following 4-h treatment of TNF α , the cell-surface expression of VCAM-1 and E-selectin

25 was measured by using flow cytometry.

Effect of ceramide and Sph-1-P on adhesion protein expression

HUVECs were treated with an increasing concentration of C₂-ceramide with or without TNF α (100 U/ml), or an increasing concentration of Sph-1-P for 4 h. The cell-surface expression

30

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of VCAM-1 or E-selectin was measured by flow cytometry. Effect of sphingosine kinase inhibitor (DMS) on the expression of VCAM-1 and E-selectin. The cells were treated with a vehicle (Nil), DMS (5 μ M), Sph-1-P (5 μ M), C₂-ceramide (10 μ M) or TNF α (100 U/ml) for 4 h, then measure VCAM-1 or E-selectin expression. Effect of agents on E-selectin mRNA levels and the comparison between the effect of HDL and DMS. After the indicated treatment for 4 h, E-selectin mRNA levels were measured by Northern blotting assay. Results shown in (D) are representative of three similar experiments.

Effect of HDL on TNF α -induced sphingomyelin hydrolysis, ceramide generation, sphingosine kinase activation, and Sph-1-P production

HUVECs were labelled with [³H]sphingomyelin was measured at the desired time point of TNF α treatment. The unlabelled cells were treated with HDL and/or TNF α as indicated, cells were lysed to measure ceramide levels and sphingosine kinase activity, respectively.

The cells were permeabilized to measure the production of Sph-1-P *in vivo*.

Effect of HDL and sphingosine kinase pathway on ERK and NF- κ B activation

After the cells were treated with an agent for 30 min, ERK activities were assayed with myeline basic protein (MBP) as substrate after immunoprecipitation with antibodies against p42/p44^{MAPK} (Berra *et al.*, 1995; Li *et al.*, 1996; Lee *et al.*, 1997). The kinase reaction products were separated on 10% SDS-polyacrylamide gels. Bar graph depicting ERK activities were quantified by Phosphoimager. NF- κ B binding activity was measured by electrophoretic mobility shift assay after 30 min treatment as indicated. The specific NF- κ B binding complexes were identified by the super-shift gel assay with anti-p50 and anti-p65 antibodies and by competition analyses with the addition of a 50-fold molar excess of unlabelled NF- κ B oligonucleotides.

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EXAMPLE 3**Sphingosine kinase signalling pathway**

To determine the role of sphingolipid metabolites in adhesion protein expression, either a
5 cell-permeable form of ceramide (C_2 -ceramide) or sphingomyelinase that generates
endogenous ceramide, or Sph-1-P were added to endothelial cells. C_2 -ceramide (Figure 2A)
or sphingomyelinase were poor stimulators of E-selectin and VCAM-1 expression, reaching
levels that were less than 10% of that stimulated by TNF- α . By contrast Sph-1-P was a
potent and dose-dependent inducer of E-selectin and VCAM-1, reaching levels at 5 μ M that
10 were approximately equivalent to 100U/ml TNF- α (Figure 2B). The potency of Sph-1-P
was further shown by its induction of E-selectin mRNA (Figure 2D). To examine the role
of Sph-1-P was inhibited by the competitive inhibitor of sphingosine kinase, N,N-
dimethylsphingosine (DMS). DMS decreased the TNF- α -induced adhesion protein
expression and mRNA levels by between 50 and 70% (Figures 2C and D). This
15 demonstrates that sphingose kinase activation is an important event in the TNF- α action.
As a control, the same concentration of DMS inhibited sphingosine kinase activity and Sph-
1-P formation by >90% induced either by TNF- α or PMA in endothelial cells, PMA has
been shown to activate sphingosine kinase via protein kinase C activation (7). In contrast
to its effect on TNF- α DMS did not prevent Sph-1-P induced adhesion protein expression
20 (Figure 2C), indicating a specific effect of DMS on the generation of Sph-1-P. These results
show a new signalling pathway for TNF- α -induced adhesion molecule expression.
Exogenous cell-permeable ceramide was a potent inhibitor (by >60%) of TNF- α induced
adhesion protein expression (Figure 2A).

25

EXAMPLE 4**HDL inhibition of sphingosine kinase signalling**

Figure 3A shows that TNF α stimulation of HUVECs rapidly reduced cellular sphingomyelin
content to 40% of control within 30 min with return to near basal levels by 2 h. In parallel,
30 the cellular ceramide levels were rapidly increased (approximately 2 fold) peaking at 30 min

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after TNF α treatment (Figure 3B). Treatment of endothelial cells with HDL had a potent effect: it delayed the reversion of post TNF α sphingomyelin levels to base line and sustained the increased ceramide levels after TNF α stimulation.

5 TNF α stimulation of HUVECs caused a rapid and transient increase in cytosolic sphingosine kinase activity, reaching a maximum of $165 \pm 13\%$ ($p < 0.01$) of basal within 5 min. HDL pretreatment profoundly inhibited both the amplitude and duration of TNF α -induced sphingosine kinase activation (Figure 3C). The production of Sph-1-P was induced in parallel with the sphingosine kinase activity. HDL treatment again substantially blunted the
10 amplitude and duration of Sph-1-P formation (Figure 3D).

EXAMPLE 5

HDL inhibition of ERK cascade

15 Figure 4A shows that both TNF α and Sph-1-P were approximately equipotent in stimulating ERK activities, whereas C₂-ceramide did not. Treatment with DMS inhibited TNF α -activated ERK by 50% ($p < 0.02$), showing a role for sphingosine kinase in the TNF α -activated ERK signal cascade. Preincubation of HUVECs with HDL also reduced TNF α -stimulated ERK activation, consistent with its effect on reducing cellular levels of Sph-1-P
20 (Figure 4A, bar 4).

EXAMPLE 6

HDL inhibition of NF- κ B activation

25 To measure the NF- κ B binding activity, electrophoretic mobility-shift assays were performed. Nuclear extracts were prepared from HUVECs treated for 30 min with vehicle or the indicated agents. The double-stranded oligonucleotides used as a probe in these experiments included 5'-GGATGCCATTGGGGATTTCCTCTTTACTGGATGT-3' (SEQ ID NO: 1) which contains a consensus NF- κ B binding site in E-selection promoter that is
30 underlined. Gel mobility shift of a consensus NF- κ B oligonucleotide was performed by

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incubating a ^{32}P -labelled NF- κB probe with 4mg of nuclear proteins. The Sph-1-P induced formation of NF- κB specific complexes were similar us that induced by $\text{TNF}\alpha$. These specific DNA-protein complexes were completely abolished by addition of a 50-fold molar excess of unlabeled E-selectin NF- κB oligonucleotides. The specificity of NF- κB binding complex was further identified by the super-shift analyses. Anti-p50 and Anti-p65 polyclonal antibodies (purchased from Santa Cruz Biotechnology, CA) were added prior to addition of radiolabeled NF- κB probe. The same phenotype of gel retardation was shown in the gel shift assay after the the preincubation of nuclear extracts from both Sph-1-P and $\text{TNF}\alpha$ -treated cells with the antibodies. Electrophoretic mobility shift assay shows that treatment of HUVECs with Sph-1-P induced a significant nuclear NF- κB accumulation (Figure 4B). The composition of Sph-1-P induced NF- κB specific protein-DNA complexes were identical to that induced by $\text{TNF}\alpha$ which was revealed to be p50/p65 heterodimer by antibody supershift assay and by competition analyses (Figure 4B, the bottom). Treatment of cells with HDL or DMS markedly inhibited the $\text{TNF}\alpha$ -induced activation of NF- κB by 45-60%, but did not inhibit that induced by Sph-1-P.

EXAMPLE 7

RNA preparation

Total RNA was prepared from HUVECs that was treated with the indicated agents for 4h. Equal aliquots of total RNA (12 μg) were electrophoresed in a 1% formaldehyde gel and transferred to nylon membrane. The blots were hybridized with $\alpha^{32}\text{P}$ -labeled E-selection cDNA probe. The mRNA levels of E-selectin were quantified by Phosphoimager and normalised to radiolabeled GAPDH probe.

EXAMPLE 8

Spingomyelin measurement

To measure sphingomyelin, the HUVECs were labeled with [^3H]serine (5 $\mu\text{Ci/ml}$) for 48h and preincubated with or without HDL for another 6h. The cells were then washed three

- 35 -

times and incubated for additional 2h in the presence or absence of HDL. After the treatment with TNF α for the indicated times, cellular lipids were extracted and resolved by thin-layer chromatography (TLC) with chloroform:methanol:acetic acid:water (50:30:8:5, v/v). Sphingomyelin spots were visualized by fluorography, quantitated by scintillation spectrometry, and normalized by radioactivity recovered in total cellular lipids. In the uptake of [3 H]serine there are no significant differences between the cells that were preincubated with HDL and with HDL. To measure ceramide levels, cellular ceramide was extracted and quantified with the diacylglycerol kinase reaction (Kolesnick, 1991; Hannun & Bell, 1993). Sphingosine kinase activity was measured *in vitro* as previously described with some modification (Matti *et al.*, 1994; Ghosh *et al.*, 1994; Choi *et al.*, 1996). Cells were lysed by passing through 26½ G syringe 6 times in 0.1 M HEPES buffer (pH 7.2) containing 10mM MgCl $_2$, 20% glycerol, 1mM mecaptoethanol, 1mM EDTA, 20μM ZnCl $_2$, 1mM Na $_3$ VO $_4$, 15mM NaF, 10μg/ml leupeptin and aprotinin, 1mM PMSF and 0.5mM 4-deoxypyridoxine, Cytosolic fractions were prepared by ultracentrifugation at 105,000xg for 90min. Sphingosine kinase activity was measured by incubating the supernatant with 20mM sphingosine-BSA complex and [γ^{32} P]ATP (1mM, 5μCi/ml) for 15min at 37°C. Labeled lipids were extracted and separated by TLC along with Sph-1-P standard. Radioactivity of the spot corresponding to Sph-1-P was quantified using Phosphoimager system. The measurement of Sph-1-P formation *in vivo* was performed as previously described (Olivier, 1996). The cells were permeabilized for 15min at 37°C in hypotonic buffer (25mM HEPES, 5mM MgCl $_2$, 20μM ZnCl $_2$, 20μM Na $_3$ VO $_4$, 10μg/ml leupeptin and aprotinin, 1mM PMSF and 0.5mM 4-deoxypyridoxine, pH 7.2) with sphingosine (10μM) and [γ^{32} P]ATP (1μM, 10μCi/ml). Lipids were extracted and resolved by TLC, and [γ^{32} P]Sph-1-P was quantified as described above.

25

EXAMPLE 9

A multi-well assay for sphingosine-kinase activity.

Flashplates were coated with lipid substrate by adding 200 μl of a mix of 30 phosphatidylcholine (19.5 μg/ml), phosphatidylserine (5.5 μg/ml), and sphingosine (25

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$\mu\text{g/ml}$) in methanol and evaporating at 30°C under N_2 for 3 hours. A source of sphingosine kinase is added in a buffer consisting of 50mM HEPES pH 7.2/20% glycerol/10mM MgCl_2 /1mM DTT/20 μM ZnCl_2 /1mM Na_3VO_4 /15mM NaF/0.5mM 4-deoxypyridoxine along with [^{33}P]- γ -ATP (Bresatech, 1 $\mu\text{Ci/well}$ /200 μM) and incubated for up to 2 hours at 37°C .
5 The plates are then washed with 400 μl of 50mM sodium pyrophosphate, 4 times and radioactivity was measured in a scintillation counter (Top count, Packard).

EXAMPLE 10

Preparation of homogenates of tissues for survey of sphingosine kinase activity

10

Tissues were collected from freshly sacrificed animals and rapidly frozen in liquid nitrogen, 2-5 grams of wet tissue were homogenized with a Polytron tissue homogenizer in 2 volumes of 50mM Tris pH 7.4/20% glycerol/1mM DDT/1mM EDTA, containing a protease inhibitor cocktail (Boehringer-Mannheim). The material was subjected to centrifugation at
15 7500g for 30 minutes to remove large fragments. The resulting supernants were further centrifuged at high speed (100,000 X g for 1 hour) to generate "supernatant" and "pellet" fractions.

EXAMPLE 11

20

The multi-well assay is linear over time and specific.

Figure 7 illustrates the results of assaying for sphingosine kinase activity from a lysate made from human umbilical endothelial cells. It can be seen that the signal increases linearly over a period of approximately 400 minutes. This signal is specific to sphingosine kinase since
25 omitting sphingosine from the lipid mix used to coat the plates reduces the resulting signal to background levels. This demonstrates that it is sphingosine, and not some other component of the lipid or assay mix, that is being phosphorylated in the assay. This is remarkable since this assay was done with a crude lysate which would be expected to contain a number of lipid and protein kinases.

30

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EXAMPLE 12**Sphingosine-kinase agonist and antagonist activity measurement**

Plates are prepared as described in Example 9. Sphingosine kinase (prepared in buffer
5 together with ^{33}P -ATP as described in Example 9) is added in the presence of an agent to be
tested for antagonistic or agonistic activity. The agent is added to a level of enzyme activity
yielding a linear response with respect to time from 0 - 3 hours of incubation. Plates are
incubated for two hours at 37°C , washed as in Example 9 and phosphorylation of
sphingosine is determined by scintillation counting.

10

Inhibition (antagonistic activity) is detected as diminished incorporation of ^{33}P and stimulation
(agonistic activity) is detected as increased incorporation of ^{33}P relative to a control
incubation containing an equivalent amount of the vehicle solvent used to deliver the agents
tested.

15

Those skilled in the art will appreciate that the invention described herein is susceptible to
variations and modifications other than those specifically described. It is to be understood
that the invention includes all such variations and modifications which fall within its spirit
and scope. The invention also includes all of the steps, features, compositions and
20 compounds referred to or indicated in this specification, individually or collectively, and any
and all combinations of any two or more of said steps or features.

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CLAIMS

1. A method of modulating cellular activity in a mammal said method comprising a administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway.
2. A method according to claim 1 wherein said component is sphingosine kinase.
3. A method according to claim 1 wherein said component is sphingosine-1-phosphate.
4. A method according to claim 1 wherein said component is sphingosine kinase and sphingosine-1-phosphate.
5. A method according to any one of claims 1 to 4 wherein said cellular activity is adhesion molecule expression.
6. A method according to claim 5 wherein said adhesion molecule expression is endothelial molecule expression.
7. A method according to claim 6 wherein said modulation is a decrease in endothelial molecule expression.
8. A method according to any one of claims 1-7 wherein said agent is HDL.
9. A method of modulating cellular activity in a mammal said method comprising administering to said mammal an effective amount of one or more components of a sphingosine kinase signalling pathway or functional equivalent thereof.
10. A method according to claim 9 wherein said component is sphingosine kinase.

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11. A method according to claim 9 wherein said component is sphingosine-1-phosphate.
12. A method according to any one of claims 9 to 11 wherein said cellular activity is adhesion molecule expression.
13. A method according to claim 12 wherein said adhesion molecule expression is endothelial cell adhesion molecule expression.
14. A method according to claim 13 wherein said modulation is a decrease in endothelial molecule expression.
15. A method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of one or more components of a sphingosine kinase signalling pathway wherein said modulation results in modulation of adhesion molecule expression.
16. A method according to claim 15 wherein said disease condition is coronary heart disease.
17. A method according to claim 15 or 16 wherein said component is sphingosine kinase.
18. A method according to claim 15 or 16 wherein said component is sphingosine-1-phosphate.
19. A method according to claim 15 or 16 wherein said component is sphingosine kinase and sphingosine-1-phosphate.
20. A method according to any one of claims 15-19 wherein said adhesion molecule expression is endothelial molecule expression.

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21. A method according to claim 20 wherein said modulation is a decrease in adhesion molecule expression.
22. A method according to any one of claims 15-21 wherein said agent is HDL.
23. A method of treatment or prophylaxis of a disease condition involving inflammatory mechanisms in a mammal said method comprising administering to said mammal an effective amount of one or more components of a sphingosine kinase signalling pathway or functional equivalent thereof.
24. A method according to claim 23 wherein said disease condition is coronary heart disease.
25. A method according to claim 23 or 24 wherein said adhesion molecule expression is endothelial molecule expression.
26. A method according to claim 25 wherein said modulation is a decrease in adhesion molecule expression.
27. Use of an agent capable of modulating the activity of one or more components of a sphingosine kinase signalling pathway in the manufacture of a medicament for the modulation of cellular activity in a mammal.
28. Use according to claim 27 wherein said cellular activity is adhesion molecule expression.
29. Use of one or more components of a sphingosine kinase signalling pathway or functional equivalent thereof in a manufacture of a medicament for the modulation of cellular activity in a mammal.

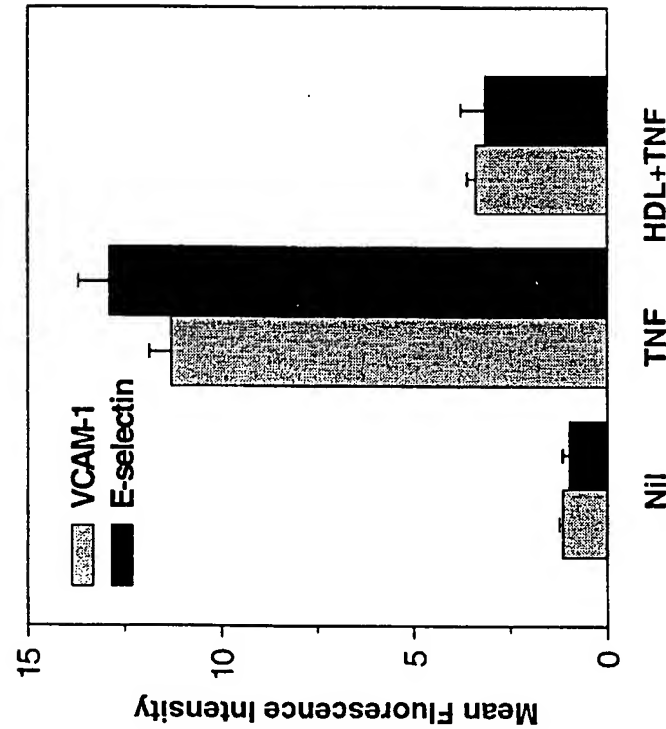
- 43 -

30. Use according to claim 29 wherein said cellular activity is adhesion molecule expression.
31. Agents for use in modulating one or more components of a sphingosine kinase signalling pathway wherein modulating said components modulates cellular activity in a mammal.
32. The agent according to claim 31 wherein said cellular activity is adhesion molecule expression.
33. A composition comprising one or more components of a sphingosine kinase signalling pathway or functional equivalent thereof for use in modulating cellular activity.
34. The composition according to claim 33 wherein said cellular activity is adhesion molecule expression.
35. A pharmaceutical composition comprising an agent capable of modulating one or more components of a sphingosine kinase signalling pathway wherein said modulation results in modulation of cellular activity together with one or more pharmaceutically acceptable carriers and/or diluents.
36. A pharmaceutical composition comprising one or more components of a sphingosine kinase signalling pathway or functional equivalent thereof together with one or more pharmaceutically acceptable carriers and/or diluents.
37. A pharmaceutical composition according to claim 34 or 35 wherein said cellular activity is adhesion molecule expression.

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38. A method of detecting an analyte structurally or functionally reactive with a lipid, said method comprising the steps of contacting either:
- (i) said analyte which analyte is radio labelled; or
 - (ii) said analyte and a reporter molecule which reporter molecule is radio labelled, said analyte being contacted simultaneously with or separately to said reporter molecule;
- with said lipid in the presence of a scintillant for a time and under conditions sufficient for a lipid-radio label complex to form and to excite said scintillant, and detecting said excited scintillant.
39. A method according to claim 38 wherein said lipid and said scintillant are coupled to a solid support.
40. A method according to claim 38 or 39 wherein said lipid is sphingosine.
41. A method for detecting an analyte exhibiting sphingosine kinase activity, said method comprising the steps of contacting said analyte and ^{33}P -ATP with sphingosine in the presence of a scintillant, said analyte being contacted with said sphingosine simultaneously with or separately to said ^{33}P -ATP, for a time and under conditions sufficient for ^{33}P -sphingosine complex to form and to excite said scintillant, and detecting said excited scintillant.
42. A method according to claim 41 wherein said sphingosine and said scintillant are coupled to a solid support.
43. The method according to any one of claims 1 to 26 wherein said mammal is a human.

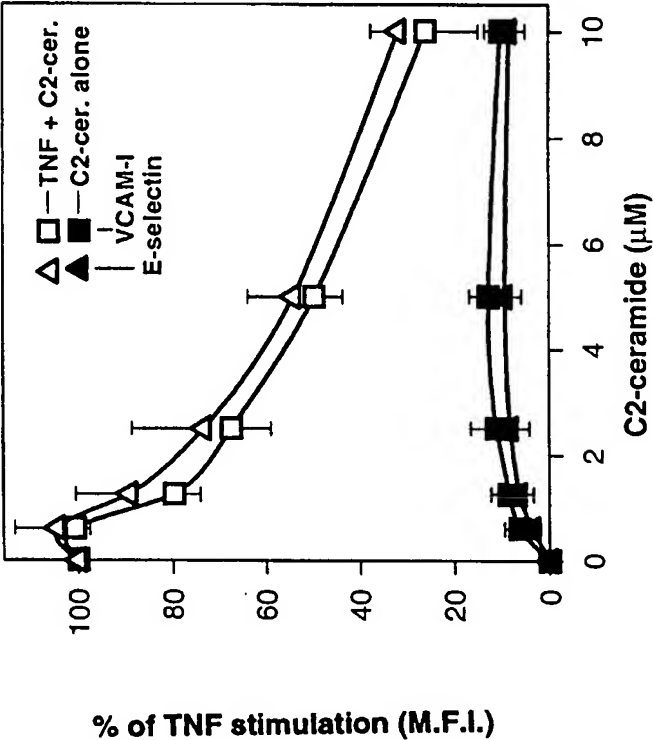
Fig.1



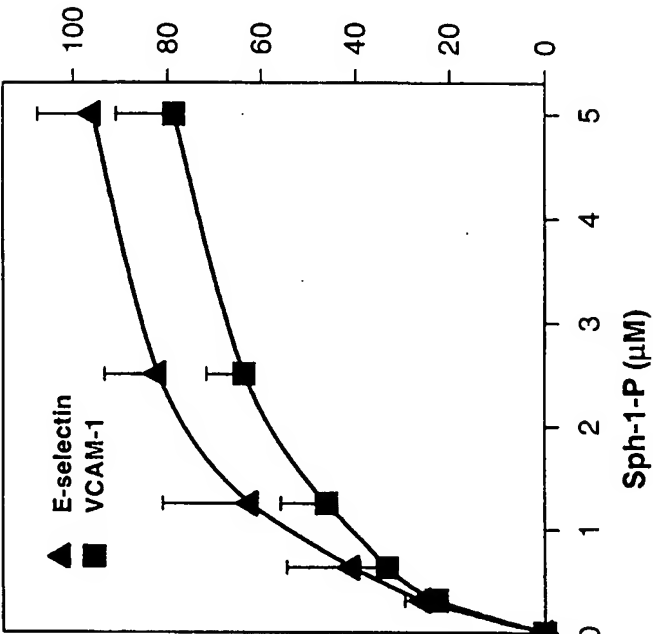
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Fig.2

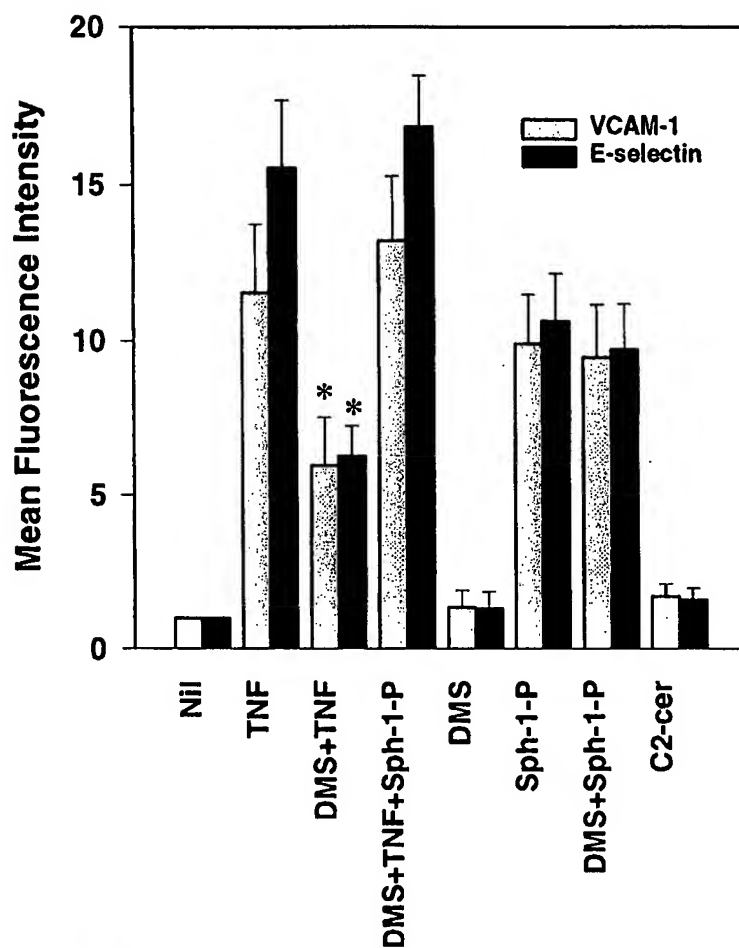
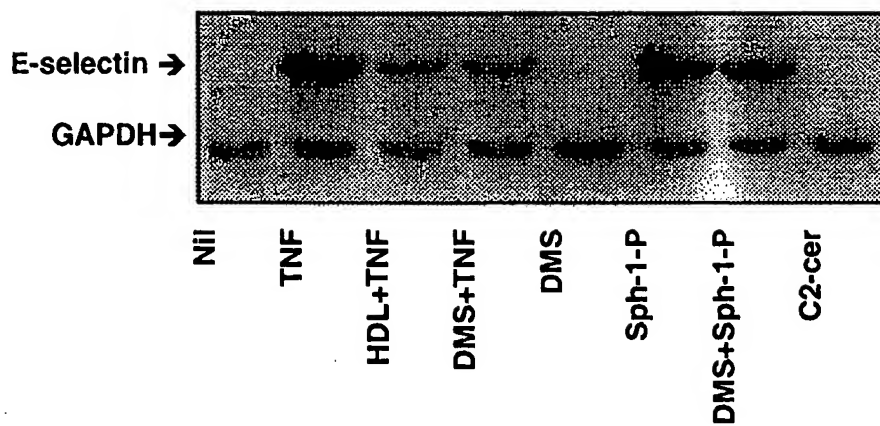
A.



B.

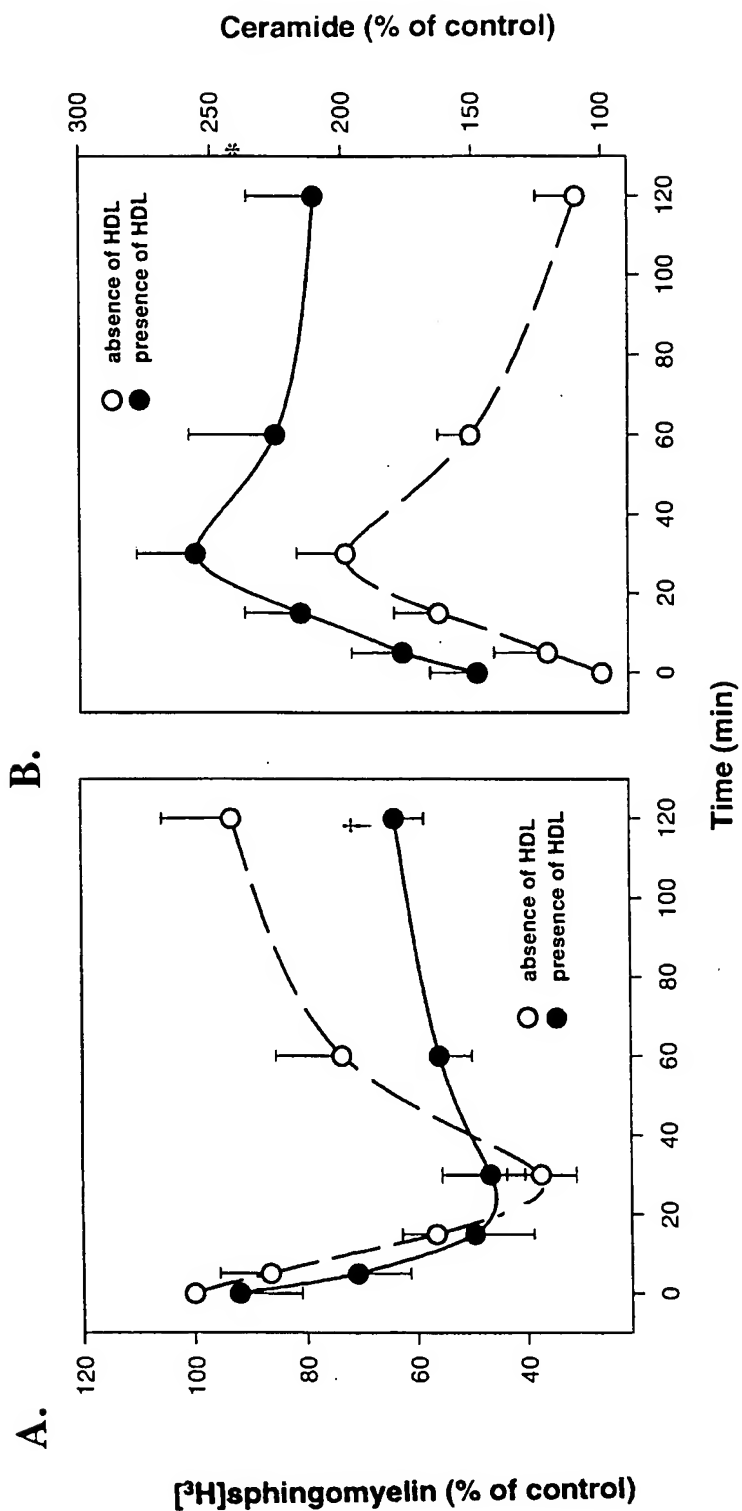


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Fig.2**C.****D.**

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Fig.3



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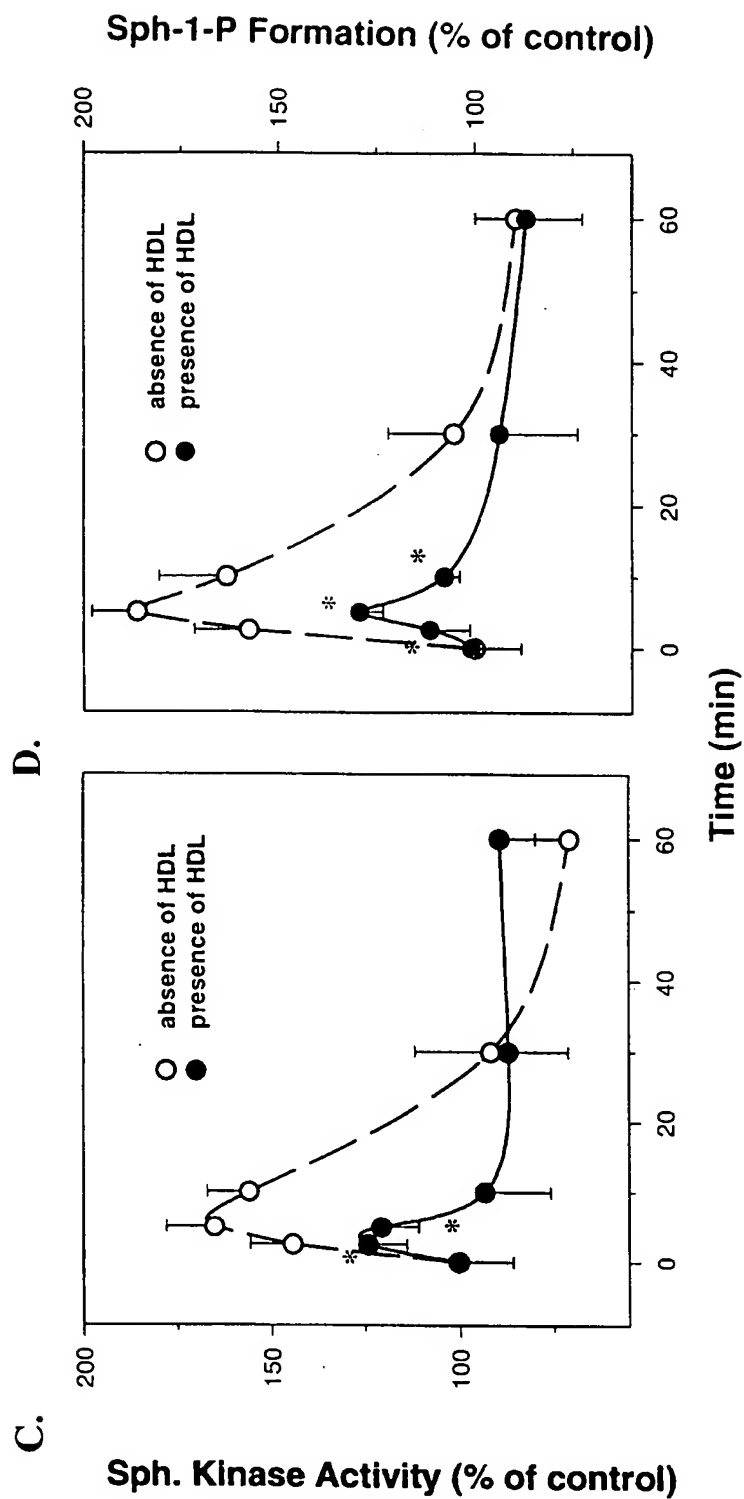
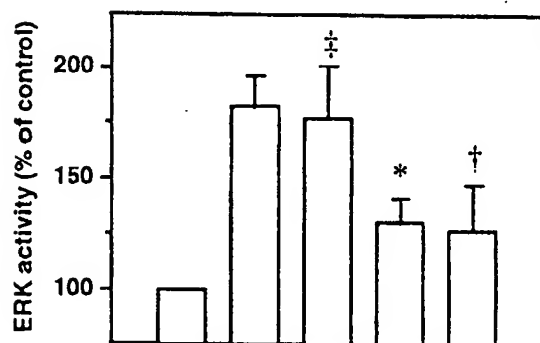
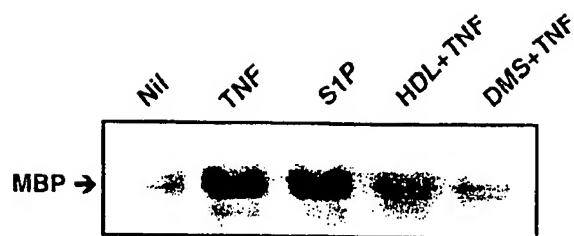


Fig.3

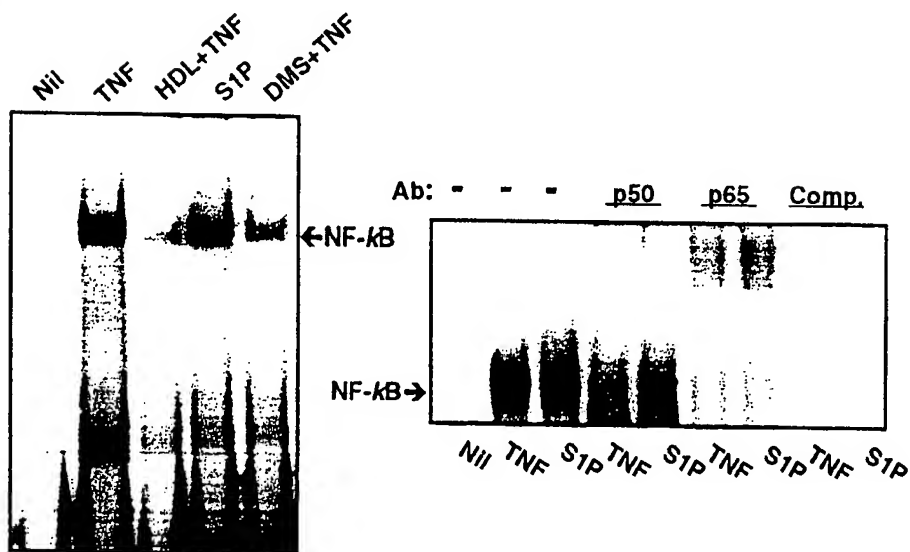
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Fig. 4.

A.

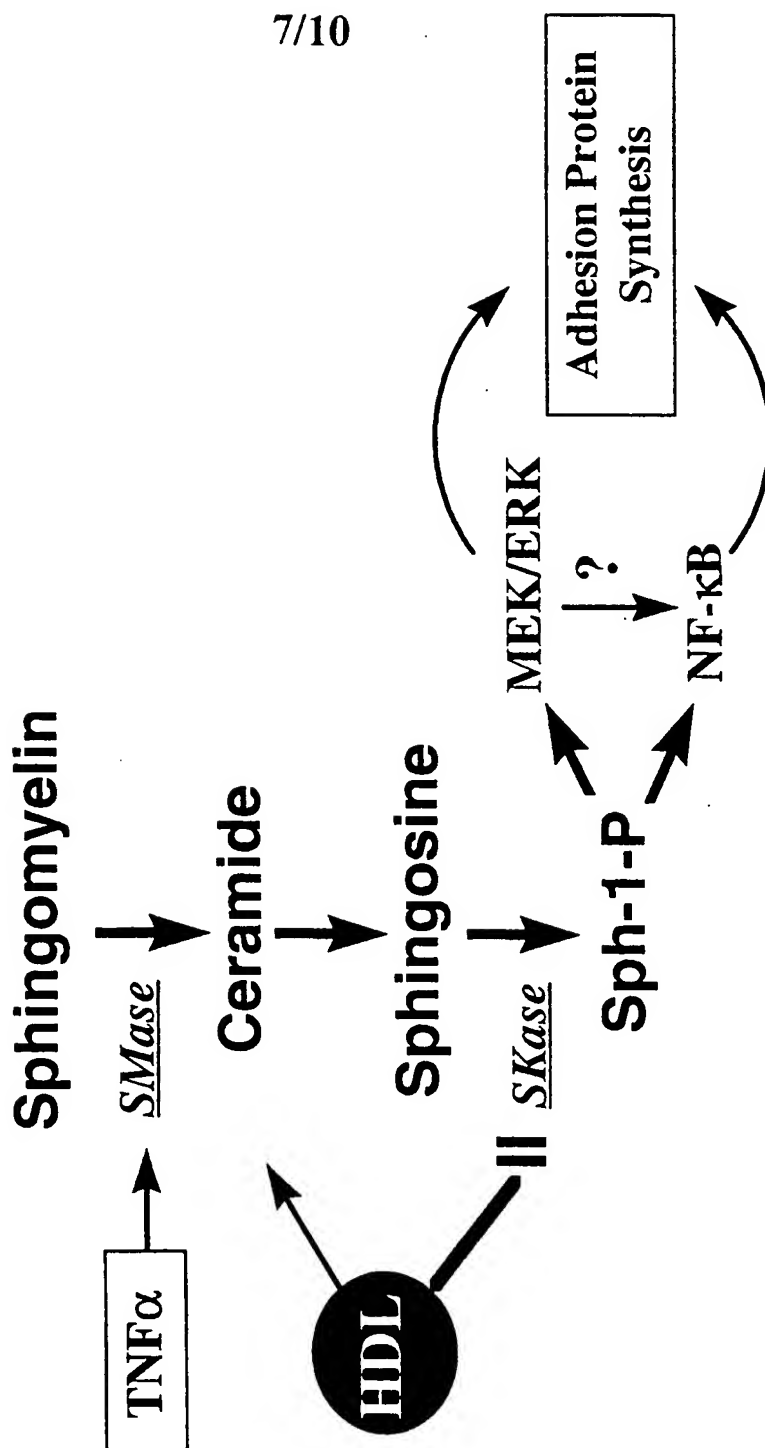


B.



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Fig.5



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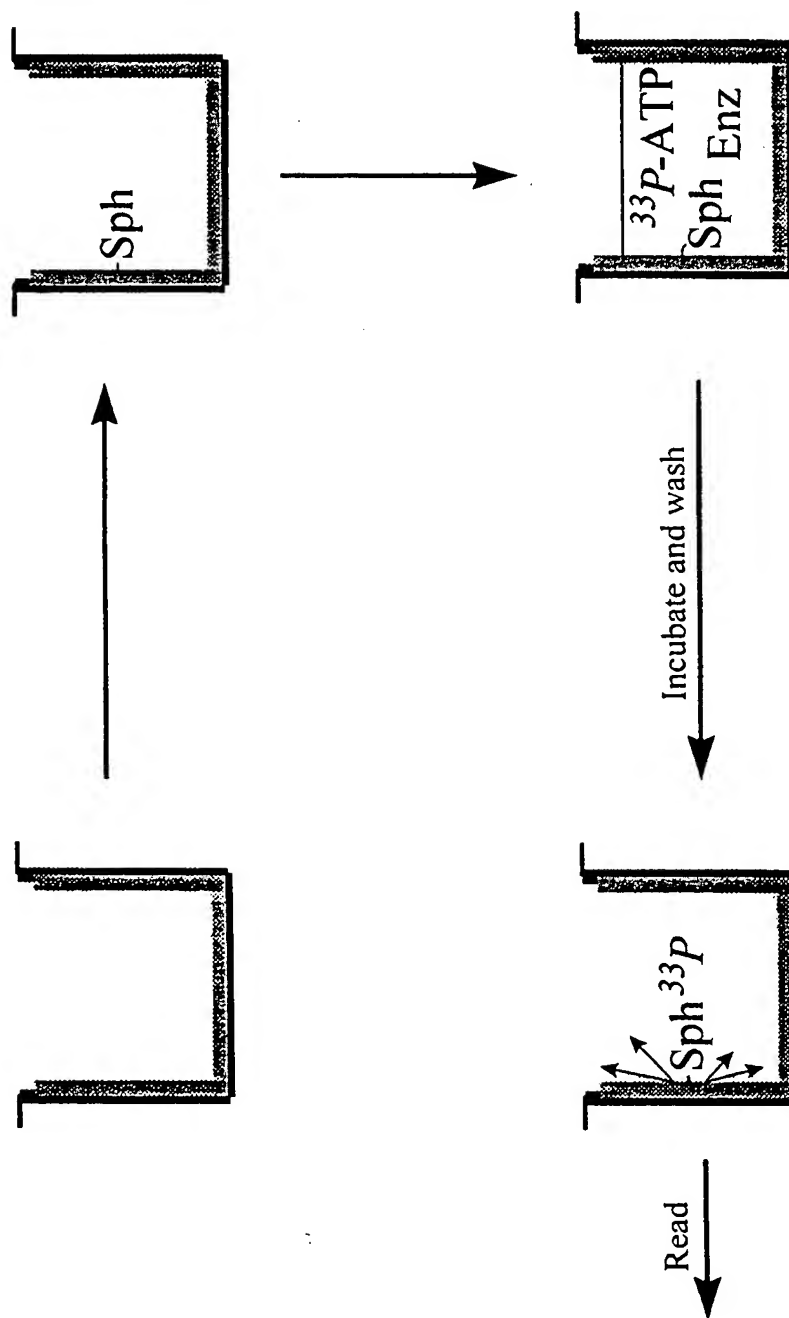


FIG 6

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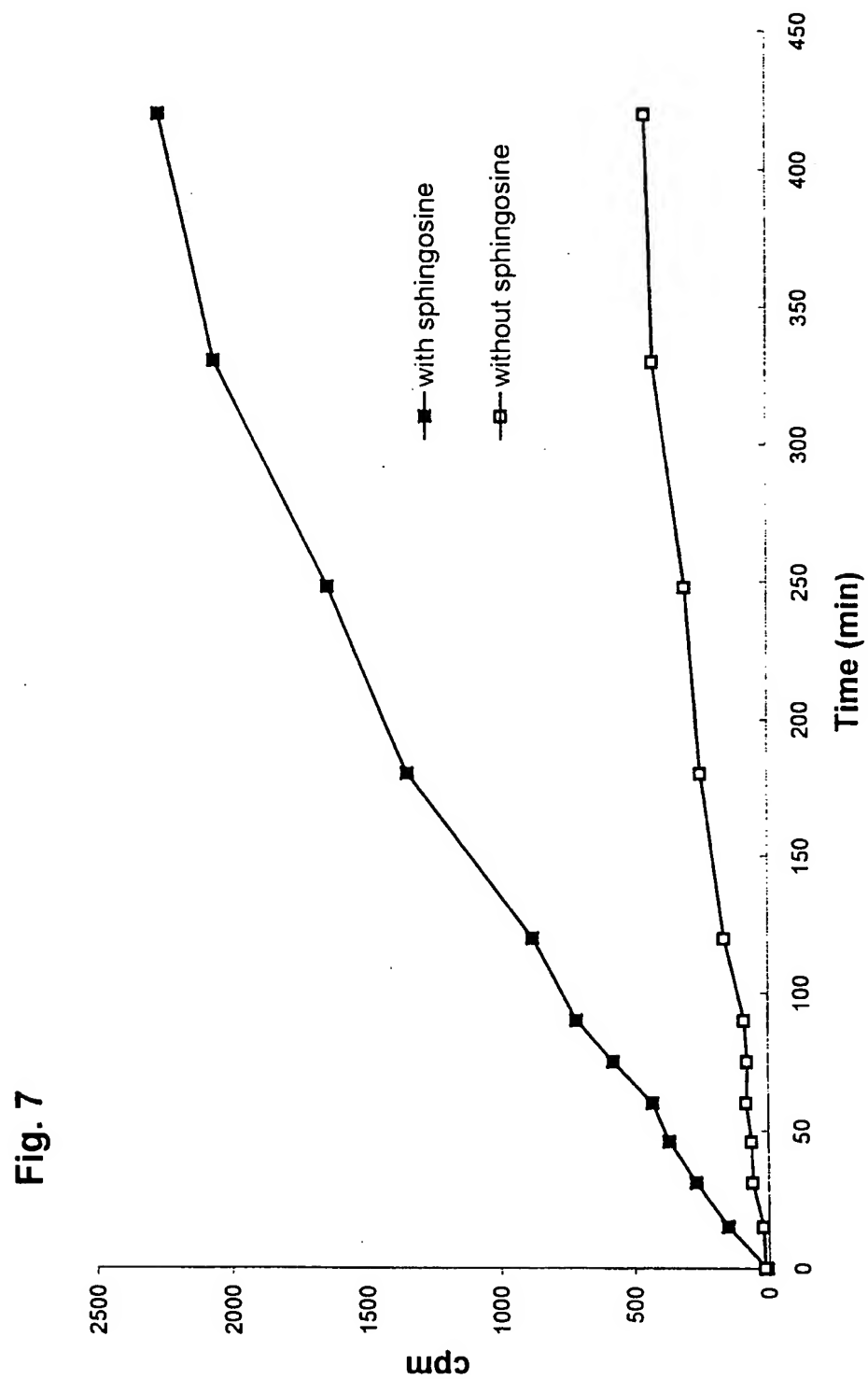
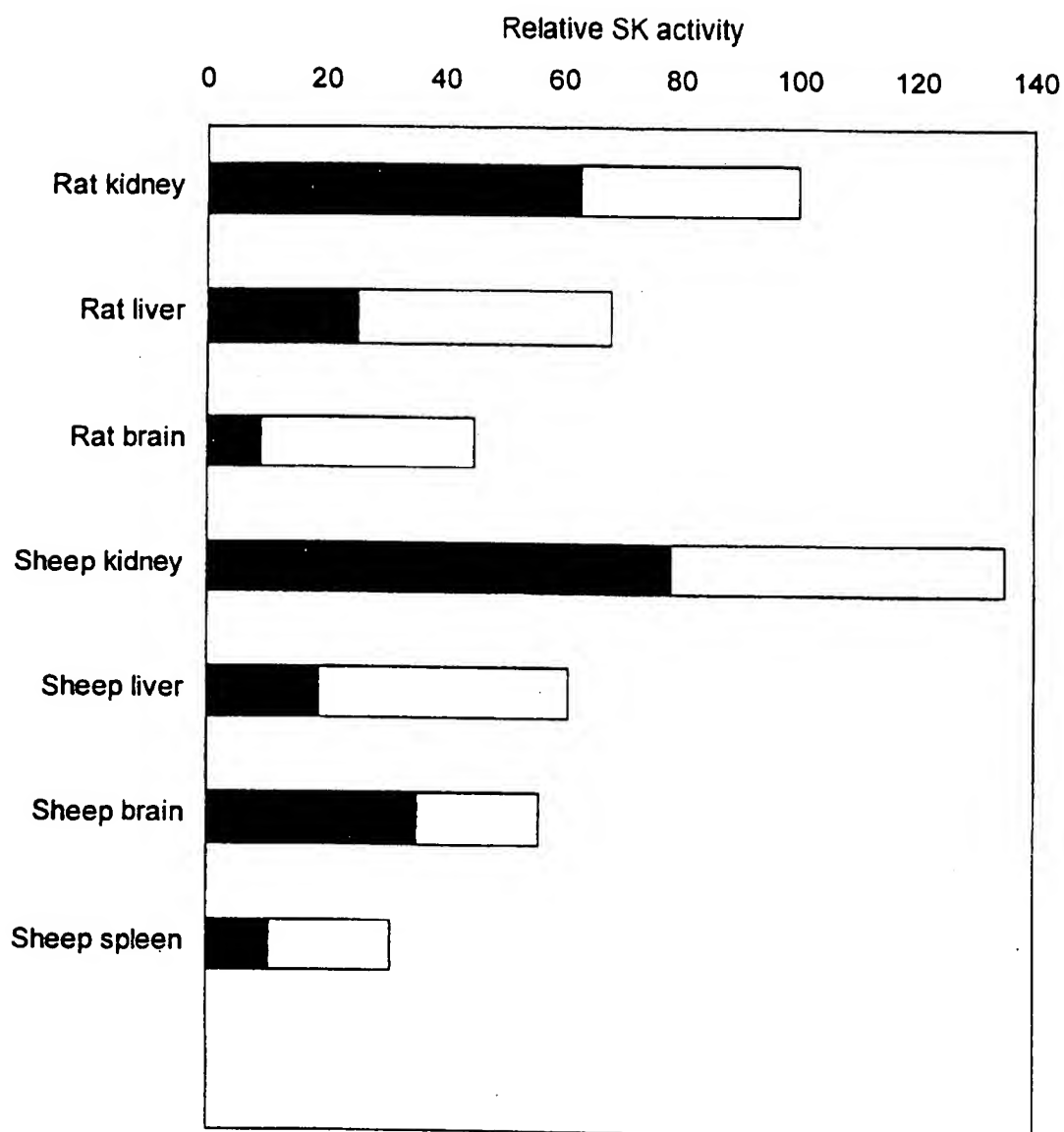


Fig. 8**10/10**

Activity in Supernatant



Activity in Pellet

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: MEDVET SCIENCE PTY LTD

(ii) TITLE OF INVENTION: A METHOD OF MODULATING CELLULAR ACTIVITY

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: DAVIES COLLISON CAVE

(B) STREET: 1 LITTLE COLLINS STREET

(C) CITY: MELBOURNE

(D) STATE: VICTORIA

(E) COUNTRY: AUSTRALIA

(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: AU PROVISIONAL

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: SLATTERY, JOHN

(C) REFERENCE/DOCKET NUMBER: JMS/dk

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: +61 3 9254 2777

(B) TELEFAX: +61 3 9254 2770

(C) TELEX: AA 31787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 nucleic acids
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATGCCATT GGGGATTTCC TCTTTACTGG ATGT

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 98/00730

A. CLASSIFICATION OF SUBJECT MATTER																						
Int Cl ⁶ : A61K 31/13, 35/14, 38/00																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols) IPC A61K and keywords below																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) high density lipoprotein, springosine, heart, atherogenesis, inflammation, coronary.																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	US 5466716 A (YASUYUKI IGARASHI et al) 14 November 1995 (see whole document)	1-7, 9-22, 23-37																				
X	WO 96/20710 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 11 July 1996 (see whole document)	1, 15, 16, 27, 31, 35																				
X	WO 95/03039 A (THE BIOMEMBRANE INSTITUTE) 2 February 1995 (see whole document)	1-7, 9-22, 23-37																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex																						
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
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"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 10 October 1998		Date of mailing of the international search report 20 NOV 1998																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer BERNARD NUTT Telephone No.: (02) 6283 2491																				

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00730

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0381514 A (THE BIOMEMBRANE INSTITUTE) 8 August 1990 (see whole document)	9, 23, 24, 29, 31, 33, 36
X	WO 90/07571 A (EMORY UNIVERSITY) 12 July 1990 (see whole document)	9, 23, 24, 29, 31, 33, 36
X	US 4766111 A (CATSIMPOOLAS, NICHOLAS) 23 August 1988 (see whole document)	9, 29, 33, 36
X	WO 88/09345 A (THE ROGOSIN INSTITUTE) 1 December 1988 (see whole document)	27, 31, 32, 35
X	WO 97/01752 A (ONCOMEMBRANE, INC) 16 January 1997 (see whole document)	38-43
P.X	Derwent Abstract accession No. 98-433849:37, Class B02, D16, JP 10182627 A (SANKYO CO LTD) 7 July 1998 (See Abstract)	1, 2, 5-7, 15-17, 20, 21, 27, 28, 31, 32, 35, 37
P.X	Patent Abstracts of Japan JP 10101630 A (SANKYO CO LTD) 21 April 1998	1, 2, 5-7, 15-17, 20, 21, 27, 28, 31, 32, 35, 37

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00730

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claims 1-37, 43 are directed to an agent or components of the sphingosine signalling pathway which affect adhesion molecule production.

Claims 38-42 are directed to detection methods, some of which have no relationship to the sphingosine kinase signalling pathway. Furthermore claims 38-42 share no special technical features with claims 1-37 and 43.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Information on patent family members

International application No.
PCT/AU 98/00730

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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